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13. ABSTRACT (Maximum 200 words) This project utilized the zinc enzyme human carbonic anhydrase II (CAII) as a paradigm for dissecting and understanding the structural basis of protein-transition metal recognition and discrimination. We have successfully redesigned the zinc binding site of CAII so that protein-metal affinity can be modulated by design, by factors of 10 from micromolar to femtomolar affinity. Additionally, our work has illuminated the structural basis of metal binding kinetics, a prerequisite for the development and deployment of a metal ion biosensor.				
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## FINAL REPORT

GRANT #: N00014-96-1-0288

PRINCIPAL INVESTIGATOR: David W. Christianson

INSTITUTION: University of Pennsylvania

GRANT TITLE: Structural Studies of Metalloprotein Design

AWARD PERIOD: 20 December 1995 - 19 November 1998

OBJECTIVE: To understand interactions responsible for protein-transition metal recognition and discrimination by redesigning the zinc binding site of carbonic anhydrase II (CAII); this work is leading to the development and deployment of a metal ion biosensor.

APPROACH: Site-specific variants of CAII are prepared in the laboratory of collaborator Carol Fierke at Duke University. These variants are crystallized in the P.I.'s laboratory at the University of Pennsylvania and subject to high resolution X-ray structure determination.

ACCOMPLISHMENTS: The structures of 30 CAII variants relevant to the design of a CAII-based biosensor have been determined. We have determined the most detailed structure-affinity relationships known for any zinc metalloenzyme. Our work has focused on (a) direct metal ligands, (b) indirect metal ligands (i.e., those that accept hydrogen bonds from direct metal ligands), and (c) hydrophobic residues that surround the protein-zinc binding site. Highlights of this work include the structure-based engineering of the tightest protein-zinc binding site: the T199C variants exhibits femtomolar zinc affinity. Additionally, we are now capable of engineering protein-metal affinity by factors of 10 from the micromolar range to the femtomolar range.

Substitution of direct metal ligands H94, H96, and H119 by negatively-charged cysteine, aspartate, and glutamate residues typically maintains the tetrahedral zinc coordination polyhedron, including the zinc-bound solvent molecule. However, these variants usually exhibit compromised affinity and catalytic activity, and the pKa value for zinc-bound solvent is dramatically increased. This is due to the perturbation of the electrostatic environment of the metal ion. Introduction of a new negative charge from the protein compromises the ability of the metal ion to stabilize bound hydroxide ion, which results in an elevated pKa value. Accordingly, introduction of neutral asparagine and glutamine ligands in place of H119 results in CAII variants that retain substantial catalytic activity and exhibit near-normal pKa values for zinc-bound solvent.

Substitution of indirect zinc ligands results in more subtle effects on structure-affinity relationships. The carboxamide side chain of Q92 accepts a hydrogen bond from zinc ligand H94. The structure of the Q92E variants reveals an essentially isosteric replacement of the carboxamide side chain by the carboxylate side chain. However, the pKa of zinc-bound solvent is slightly elevated, due to the perturbation of the electrostatic environment of the metal ion. The introduction of a new negative charge even one residue removed from the metal ion affects the biological role of the metal ion in stabilizing its bound hydroxide ion. In an unusually dramatic example, the isosteric E117Q substitution results in severely-compromised activity (E117 accepts a hydrogen bond from zinc ligand H119). Unexpectedly, the structure of the E117Q variant suggests that this substitution stabilizes H119 as the negatively-charged histidinate anion, reflected by the corresponding increase in the pKa value for zinc-bound solvent.

Recent studies have focused on multiple-site CAII variants with amino acid substitutions in the beta-strand containing metal ligands H94 and H96. Structural comparison of the metal-bound and metal-free forms of selected double- and triple-site variants reveal conformational changes upon metal binding that are responsible for altered and more favorable metal binding kinetics. These CAII variants in particular are well-suited for application in a real-time CAII-based zinc biosensor, and work to this end is continuing in the laboratories of Carol Fierke (Duke University) and Richard Thompson (University of Maryland).

We have also explored the structure-based design and optimization of molecules that will transduce a signal upon CAII-zinc association. As part of this effort, we have determined the structure of the CAII-dansylamide complex (dansylamide was the prototypical signal transduction molecule employed by Richard Thompson in early zinc sensing experiments using CAII). Intriguingly, dansylamide is bound in a conformation previously unobserved for any sulfonamide inhibitor of CAII, and this has substantial implications for the structure-assisted design of transduction molecules for the CAII-based biosensor. Additionally, we designed and synthesized a fluorescein-sulfonamide chromophore capable of measuring nanomolar zinc concentrations in aqueous solution using fluorescence anisotropy.

**SIGNIFICANCE:** Our structure-based redesign experiments with CAII yield valuable information regarding structure-stability relationships of zinc binding sites in proteins. Our collaborators Carol Fierke (Duke University) and Richard Thompson (University of Maryland School of Medicine) are now

exploiting this information to optimize the molecular properties of the CAII-based biosensor.

PATENT INFORMATION: A patent application has been filed jointly with Richard Thompson at the University of Maryland on the use of CAII as a metal ion biosensor using a fluorescence anisotropy sensing scheme.

AWARD INFORMATION: Pfizer Award in Enzyme Chemistry from the American Chemical Society, 1999.

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